Master's thesis

Isolation and characterization of haloarchaea from high salinity environment

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의 분리 및 특징 규명

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Isolation and characterization of haloarchaea from high salinity environment

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ABSTRACT

Archaea is one of the three major domains of biological classification system, and this domain comprises major phyla: *Euryarchaeota*, *Crenarchaeota*, *Thaumarchaeota*, *Nanoarchaeota*, *Korarchaeota*, *Pacearchaeota*, *Woesearchaeota*, *Parvachaeota* and *Aigarchaeota*. Extremely halophilic archaea, often called as 'Haloarchaea', belong to the phylum *Euryarchaeota*. Haloarchaea is require at least 1.5 M NaCl for growth. Most of halophilic archaea are found in various hypersaline environments including solar salterns and salt lakes. The present study is about isolation and characterization of haloarchaea from Gomso solar saltern known as a representative high salinity environment in Korea. To obtain pure cultures of haloarchaea from solar saltern sediment, high salt medium (20 % NaCl) was prepared, and number of diverse haloarchaea were obtained. Among the pure colonies, one strain as a novel strain and seven strains as non-reported strains of haloarchaea were obtained. These were tentatively identified based on their physiological traits and 16S rRNA gene sequence similarity. All of these halophilic archaea belonged to *Halolamina, Haloruburm, Halogeometriucm, Halobacterium,* and *Haloarcula* genera. All

strains were Gram-stain negative, red-pigmented and undergoes cell lysis in distilled water. Under anaerobic conditions no growth was observed showing inability to use nitrate as an alternative electron acceptor and all isolates required about 12-30 % (w/v, NaCl) salt for growth. Based on phenotypic, chemotaxonomic and phylogenetic properties, a novel species of the genus *Halolamina*, for which the name *Halolamina sediminis* was proposed. This case study provides basic information on microbial isolation techniques and related research for halophilic microorganisms from domestic halophilic environments, and it is a good source of information for isolating useful indigenous halophilic archaea from a variety of extreme environmental conditions.

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Chapter 1. Isolation and characterization of non-reported archaea from Korea.

1. Introduction

Biological classification system on the earth has been established by Carl Woese (Woese & Fox, 1977; Woese *et al.*, 1990) showing three domains (*Eukarya*, *Bacteria*, and *Archaea*). Firstly *Archaea* consists of two major kingdoms (Euryarchaeota and Crenarchaeota; Woese *et al.*, 1990). However now due to the development of culture independent molecular-ecological techniques including next-generation sequencing (NGS), the *Archaea* is subdivided into *Korarchaeota*, *Nanoarchaeota*, *Thaumarchaeota*, *Woesearchaeota*, *Parvachaeota*, *Aigarchaeota* and *Pacearchaeota* (Brochier-Armanet *et al.*, 2008; Castelle *et al.*, 2015; Guy & Ettema, 2011; Spang *et al.*, 2013).

Euryarchaeota comprises a physiologically diverse group of *Archaea* including methanogens, haloarchaea, acido-thermophiles, and some hyperthermophiles, while *Crenarchaeota* mostly comprises of hyperthermophiles. Interestingly, mesophilic ammonia-oxidizing archaea belong to *Crenarchaeota*, but according to cultivation conditions and genome analysis, they were reclassified into *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008). However, no culturable microorganisms have been detected in case of *Pacearchaeota*, *Woesearchaeota*, *Korarchaeota* etc., their existence was only, so far confirmed through molecular- ecological technology (e. g. NGS).

The cultivated microorganisms of *Archaea* can be divided according to their phenotypic characterisitics and phylogenetics into several major groups including: nitrifiers, hyperthermophiles, methanogens and extreme halophiles. Among the group, Extremely halophilic archaea, often called as the ‘Haloarchaea’, contains a diverse group of microorganisms that inhabits highly saline environments, such as salt lakes (Cui *et al.*, 2006; Gutierrez *et al.*, 2008; Waino *et al.*, 2000), solar salt evaporation ponds and

artificial saline habitats including the surfaces of heavily salted foods e.g. certain meats and fish (Oren, 2014, 2015). Until now, over 40 genera of archaea has been separated from environments high in salt concentration but the diversity among them was very low, only few of these genera show diversity (http://www.bacterio.net).

So far, microorganisms in various environments including extreme environments (temperature, pH, salinity) have been identified through molecular ecological techniques based on the 16S rRNA gene analysis. Cultivation and characterization of haloarchaea has been given more importance because of the presence of industrially important products (Oren, 2015). The characterizations of several enzymes isolated from haloarchaea shows their potential industrial applications, such as the presence of amylases, proteases, and nucleases. As reported earlier, some halophilic archaea (haloarchaea), from hypersaline location, has the ability to degrade aromatic compounds (Bonfá *et al.*, 2011). Hence, it is concluded that haloarchaea has the ability to play various important physiological and ecological roles in the environment.

In this study, seven haloarchaeal strains from Gomso solar saltern sediment (Korea) were isolated, characterized and phylogenetically analysed. This study shows that i can get diverse indigenous haloarchaea through the advancements and improvements of isolation and characterization techniques. It is also helpful in the successful isolation and characterization of haloarchaea from extreme indigenous environment.

2. Materials and Methods

2.1 Sampling of solar saltern sediment and cultivation

The sample was collected from a crystallizing pond of Gomso solar saltern in Jeollabuk-do Gunsan, Republic of Korea (salinity 17–23 %, temperature 35 °C and pH 7.8; 35° 35′ 44.6″ N 126° 37′ 02.5″ E, data from Park *et al.*, 2012). A 2 g solar saltern sediment sample was collected in a sterile conical tube and serially diluted in natural brine water (filter-sterilized, 0.22 μm pore size, Millipore) with 0.5% (w/v) yeast extract (Difco). After three sub-cultures in haloarchaea media, the enrichment culture was spread onto solid agar plate [containing 1.5% (w/v) agar], and incubated at 37 °C for 2 weeks under aerobic condition.

2.2 Haloarchaea culture media

For the isolation of haloarchaea, two different medias were prepared: (i). modified S-G medium [MSG; 200 g sodium chloride, 10 g yeast extract (Difco), 20 g magnesium sulfate, 7.5 g acid hydrolyzed casein, 3 g trisodium citrate, 2 g potassium chloride, 0.05 g ferrous sulfate per liter of distilled water] (ii). MH2 [200g sodium chloride, 4g casamino acid (Difco), 2g yeast extract (Difco), 2g L-glutamic acid, 2g trisodium citrate, 5g potassium sulfate, 20g magnesium chloride, 1g ammonium chloride, 1g potassium phosphate monobasic, 0.004g ferrous sulfate] were used (Sehgal & Gibbons, 1960; Ventosa *et al.*, 1982). After the autoclave, MH2 medium added to 2ml trace element solution (Widdel & Bak, 1992).

2.3 Phylogenetic analysis

For phylogenetic analysis, genomic DNA was extracted using a commercial genomic DNA extraction kit (Geneall, Republic of Korea). The 16S rRNA gene was amplified from chromosomal DNA using the universal archaeal primer set: 20F (5'-TTCCGGTTGATCCYGCCRG-3') and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (DeLong, 1992; Weisburg *et al.*, 1991). The following cycling conditions were used: 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 60 sec; and a final extension of 72 °C for 5 min. The PCR product was purified using a PCR purification kit (Cosmo Genetech, Republic of Korea) and sequenced by Cosmogenetech using primers 20F, 340F, 958R, and 1492R (DeLong, 1992; Weisburg *et al.*, 1991). Full sequence of the gene was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of species related taxa were obtained from the BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). Levels of 16S rRNA gene sequence similarity were calculated by using the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim *et al.*, 2012). The sequences were edited using the BioEdit program (Hall, 1999) and aligned using CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distances between the sequences were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by the neighbor-joining method (Saitou & Nei, 1987), maximum-likelihood method (Felsenstein, 1981), and maximum-parsimony method (Fitch, 1971) in MEGA6 (Tamura *et al.*, 2013). The resultant tree topologies were evaluated by bootstrap analyses (1000 replications).

2.4 Morphological and physiological characterization

The physiological characterization was performed with methods recommended in the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Gram staining was performed as described by Dussault (1955). Cell lysis in distilled water and different salt concentrations [2-10 % NaCl (w/v), intervals of 2 %] was determined spectrophotometrically at 600 nm (DS-11+ spectrophotometer; DeNovix) and microscopically. Optimal growth conditions and growth ranges were determined using MH2 and MSG medium as basal medium. NaCl concentrations were 5, 10, 15, 20, 25, 30 % (w/v). The temperature range for growth was determined at 10, 15, 20, 25, 30, 37, 40, 45, 50, and 60 °C in medium with optimal NaCl concentration (20-25%, w/v). The response to pH was determined by growth on MH2 and MSG medium adjusted to pH 5.0-10.0 at intervals of 0.5 pH units using following buffers: 10 mM MES for pH 5.0–6.5, 10 mM Bis-Tris propane for pH 7.0–8.5, and 10 mM CAPS for pH 9.0–10.0. The requirement for magnesium ions for growth was determined using MH2 and MSG medium prepared without $MgSO_4 \cdot 7H_2O$, and then supplemented with 10 mM Bis-tris propane and $MgSO_4 \cdot 7H_2O$ at concentrations of 0, 0.01, 0.05, 0.5, 1, 2, 5, 100, and 500 mM.

Anaerobic growth with nitrate, L-arginine, trimethylamine *N*-oxide (TMAO) and DMSO (all at 5 g per mililiter) was tested using a 20 ml stoppered tube filled with MH2 and MSG liquid medium and incubated in the dark at 37 °C for 4 weeks. It was determined by changes in turbidity (Oren *et al.*, 1997). Hydrolysis of casein and hydrolysis of gelatin was assayed as described by Smibert & Krieg (1994). To test for growth of single carbon sources, MH2 and MSG medium without casamino acid, yeast extract, acid hydrolyzed casein, L-glutamic acid and trisodium citrate was used. Various carbon sources to be tested were added to this MH2 and MSG medium at a concentration of 5 g per liter: acetate, fumarate, glucose, glycine, glycerol, lactose, lactate, maltose,

mannose, mannitol, malate, malic acid, pyruvate, sucrose, starch, succinate, sorbitol, L-arabinose, L-lysine, L-arginine and trisodium citrate. Antimicrobial susceptibilities were determined by the method of Gutiérrez *et al*. (Gutierrez *et al.*, 2008) on MH2 and MSG agar plates with antimicrobial compound discs for 4 weeks at 37 °C.

3. Results and Discussion

In this study, number of colonies were obtained. Among the pure colonies, one as a novel strain and seven as non-reported strains of haloarchaea were recogniozed. These were tentatively identified based on their physiological traits and 16S rRNA gene sequence similarity. On the basis of this result, seven strains have been classified to non-reported archaeal strains from Korea (98.2-99.8 % similarity of 16S rRNA gene sequence), except halo-7^T. These strains were classified to four genera: *Halorubrum*, *Haloarcula*, *Halogeometricum*, *Halobacterium*.

The detailed results of characterization of the strains are as follows

3.1 Genus *Halorubrum*

Euryarchaeota; *Halobacteria*; *Halobacteriales*; *Halobacteriaceae*; *Halorubrum*.

3.1.1 *Halorubrum chaoviator* meg4

The 16S rRNA sequence similarity between *Halorubrum chaoviator* meg4 and *Halorubrum chaoviator* HALO-G^T was 99.2 %. Cells were pleomorphic occurs as coccus and rods. Growth occurs at temperature range of 25–50 °C (optimum 37 °C), pH range of 6.5–8.5 (optimum pH 7.5), while grows at 12–30 % (w/v) NaCl, and needs a minimum of 50 mM magnesium. Cells showed the hydrolysis of gelatin while the nitrate reduction to nitrite was not observed. The following compounds D-glucose, D-maltose, acetate, succinate and pyruvate were utilized as sole carbon and energy sources for growth. Resistance to streptomycin, penicillin, clindamycin, tetracycline, gentamicin and ampicillin was also observed.

3.1.2 *Halorubrum chaoviator* meg5

The 16S rRNA sequence similarity between *Halorubrum chaoviator* meg5 and *Halorubrum chaoviator* HALO-G^T was 99.2 %. Cells were pleomorphic occurs as coccus and rods. Growth occurs at temperature range of 25–55 °C (optimum 37 °C), pH range of 6.5–8.5 (optimum pH 7.5), while grows at 12–30 % (w/v) NaCl, and needs a minimum of 50 mM magnesium. Cells showed the hydrolysis of gelatin while the nitrate reduction to nitrite was not observed. The following compounds D-glucose, D-maltose, acetate, succinate and pyruvate were utilized as sole carbon and energy sources for growth. Resistance to streptomycin, penicillin, clindamycin, tetracycline, gentamicin and ampicillin was also observed.

3.1.3 *Halorubrum californiense* meg10

The 16S rRNA sequence similarity between *Halorubrum californiense* meg10 and *Halorubrum californiense* SF3-213^T was 99.5 %. Cells were coccus. Growth occurs at temperature range of 25–45 °C (optimum 37 °C), pH range of 7.0–8.5 (optimum pH 7.5), while grows at 15–30 % (w/v) NaCl, and did not requires magnesium. Cells showed the hydrolysis of gelatin while the nitrate reduction to nitrite was not observed. The following compounds D-glucose, D-maltose, acetate, succinate, lactate and pyruvate were utilized as sole carbon and energy sources for growth. Resistance to streptomycin, penicillin, clindamycin, tetracycline, gentamicin and ampicillin was also observed.

3.1.4 *Halorubrum kocurii* A5

The 16S rRNA sequence similarity between *Halorubrum kocurii* A5 and *Halorubrum kocurii* BG-1^{T} was 98.2 %. Cells were rods. Growth occurs at temperature range of 25–55 °C (optimum 37 °C), pH range of 6.0–9.0 (optimum pH 7.5), while grows at 12–29 % (w/v) NaCl, and did not requires magnesium. The hydrolysis of gelatin and nitrate reduction to nitrite was not observed. The following compounds acetate, fumarate, glycine, glycerol, lactate, malate, pyruvate, sucrose, succinate, sorbitol, D-mannose, D-mannitol, L-lysine, L-arginine, malic acid and trisodium citrate were utilized as sole carbon and energy sources for growth. Resistance to streptomycin, penicillin, clindamycin, tetracycline, gentamicin and ampicillin was also observed.

3.2 Genus *Halogeometricum*

Euryarchaeota; *Halobacteria*; *Halobacteriales*; *Halobacteriaceae*; *Halogeometricum*.

3.2.1 *Halogeometricum rufum* S9-29

The 16S rRNA sequence similarity between *Halogeometricum rufum* S9-29 and *Halogeometricum rufum* RO1-4^{T} was 99.4 %. Cells were rods. Growth occurs at temperature range of 25–55 °C (optimum 37 °C), pH range of 6.0–9.0 (optimum pH 7.0), while grows at 12–30 % (w/v) NaCl, and needs a minimum of 50 mM magnesium. The hydrolysis of gelatin and nitrate reduction to nitrite was not observed. The following compounds D-glucose, D-maltose, D-mannose, L-arbinose, acetate, malate, lactate, glycerol, succinate, malic acid and trisodium citrate were utilized as sole carbon and energy sources for growth. Resistance to streptomycin, penicillin, clindamycin, tetracycline, gentamicin and ampicillin was also observed.

3.3 Genus *Halobacterium*

Euryarchaeota; *Halobacteria*; *Halobacteriales*; *Halobacteriaceae*; *Halobacterium*.

3.3.1 *Halobacterium noricense* A10

The 16S rRNA sequence similarity between *Halobacterium noricense* A10 and *Halobacterium noricense* A1^T was 99.1 %. Cells were rods. Growth occurs at temperature range of 25–50 °C (optimum 37 °C), pH range of 5.0–7.5 (optimum pH 7.0), while grows at 12–30 % (w/v) NaCl, and needs a minimum of 500 mM magnesium. The hydrolysis of gelatin and nitrate reduction to nitrite was not observed. The following compounds acetate, fumarate, glycine, glycerol, lactate, malate, pyruvate, sucrose, succinate, D-mannose, L-lysine, L-arginine and sorbitol were utilized as sole carbon and energy sources for growth. Resistance to streptomycin, penicillin, clindamycin, tetracycline, gentamicin and ampicillin was also observed.

3.4 Genus *Haloarcula*

Euryarchaeota; *Halobacteria*; *Halobacteriales*; *Halobacteriaceae*; *Haloarcula*.

3.4.1 *Haloarcula vallismortis* meg6

The 16S rRNA sequence similarity between *Haloarcula vallismortis* meg6 and *Haloarcula vallismortis* J.F.54^T was 99.3 %. Cells were rods. Growth occurs at a temperature range of 20–50 °C (optimum 37 °C), pH range of 6.0–8.5 (optimum pH 7.5), while grows at 15–30 % (w/v) NaCl, and did not requires magnesium. Cells showed the hydrolysis of gelatin while the nitrate reduction to nitrite was not observed. The following compounds D-glucose, D-maltose, acetate, succinate and pyruvate were utilized as sole carbon and energy sources for growth. Resistance to streptomycin, penicillin, clindamycin, tetracycline, gentamicin and ampicillin was also observed.

Table 1. Physiological and biochemical characteristic of halophilic archaea. All of the data is obtained from this study. All strains are Gram strain negative, and optimum growth temperature is 37°C. All of the strains utilizes acetate and succinate, while all were negative for starch and lactose utilization.

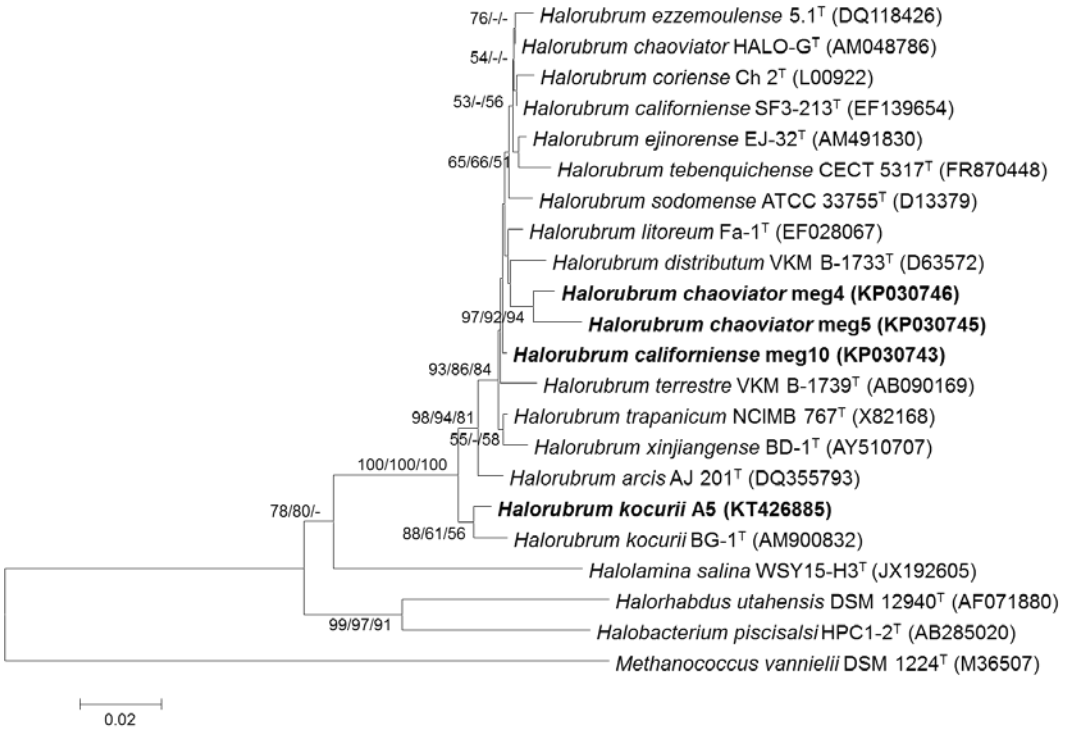
	meg4	**meg5**	**meg10**	**A5**	**S9-29**	**A10**	**meg6**
Closest related strain	*Halorubrum chaoviator* HALO-G^T	*Halorubrum chaoviator* HALO-G^T	*Halorubrum californiense* SF3-213^T	*Halorubrum kocurii* BG-1^T	*Halogeometricum rufum* RO1-4^T	*Halobacterium noricense* A1^T	*Haloarcula vallismortis* J.F.54^T
16S rRNA similarity (%)	99.2	99.2	99.5	98.2	99.8	99.1	99.3
Temperature range (°C)	25-50	25-55	25-45	25-55	25-55	25-50	20-50
pH range	6.5-8.5	6.5-8.5	7.0-8.5	6.0-9.0	6.0-9.0	5.0-7.5	6.0-8.5
pH optimum	7.5	7.5	7.5	7.5	7.0	7.0	7.5
NaCl range (%, w/v)	12-30	12-30	15-30	12-29	12-30	12-30	15-30
Mg^{2+} requirement	+	+	-	-	+	+	-
Morphology	Coccus/Rod	Coccus/Rod	Coccus	Rod	Rod	Rod	Rod
Hydrolysis:							
gelatin	+	+	+	–	–	–	+

Utilization of:							
D-glucose	+	+	+	−	+	−	+
D-maltose	+	+	+	−	+	−	+
D-mannose	−	−	−	+	+	+	−
D-mannitol	−	−	−	+	−	−	−
L-arabinose	−	−	−	−	+	−	−
L-arginine	−	−	−	+	−	+	−
L-lysine	−	−	−	+	−	+	−
Fumarate	−	−	−	+	−	+	−
Sucrose	−	−	−	+	−	+	−
Malate	−	−	−	+	+	+	−
Lactate	−	−	+	+	+	+	−
Glycine	−	−	−	+	−	+	−
Glycerol	−	−	−	+	+	+	−

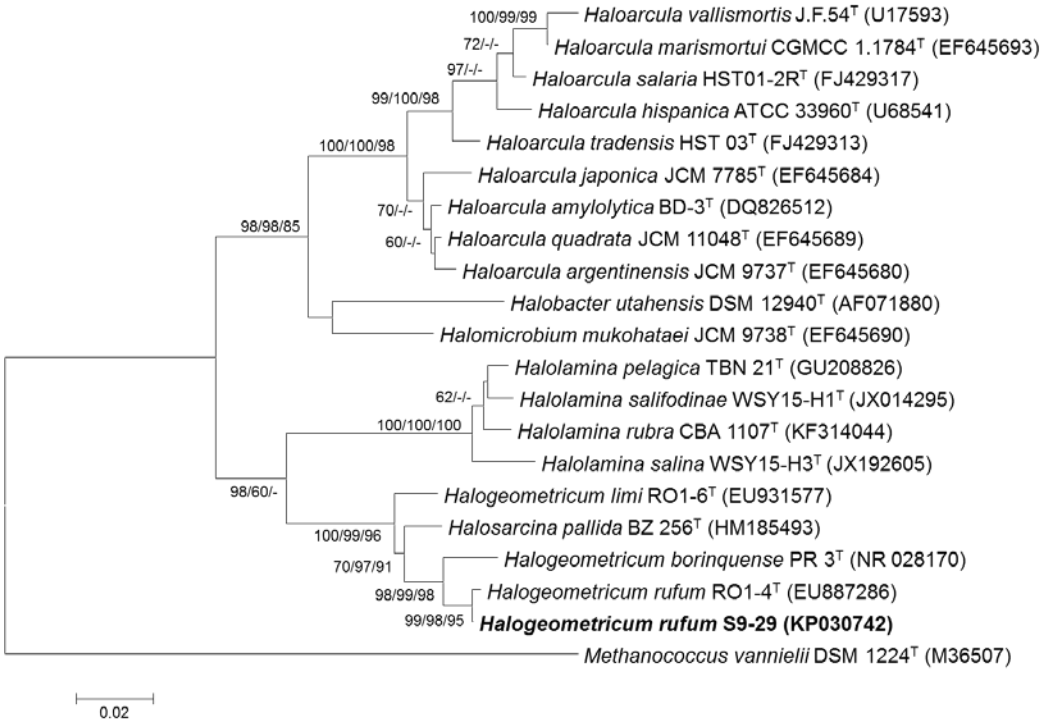
Pyruvate	+	+	+	+	−	+	+
Sorbitol	−	−	−	+	−	+	−
Malic acid	−	−	−	+	+	−	−
Citrate	−	−	−	+	+	−	−
NIBR accession No.	NIBRBA000011 5040	NIBRBA000011 5041	NIBRBA000011 5043	NIBRBAC00000 3993	NIBRBA000011 5045	NIBRBAC00000 3994	NIBRBA000011 5042

Fig. 1. Phylogenetic tree showing phylogenetic position of (A) *Halorubrum* spp. from the genus *Halorubrum*, (B) *Halogeometricum* spp. from the genus *Halogeometricum*, (C) *Halobacterium* spp. from the genus *Halobacterium*, and (D) *Haloarcula* spp. from genus *Haloarcula* based on 16S rRNA gene sequences. At branch points Bootstrap percentages ≥ 50% (based on 1000 replicates) are shown from the neighbor-joining, maximum-likelihood, maximum parsimony methods, respectively.

(A)



(B)



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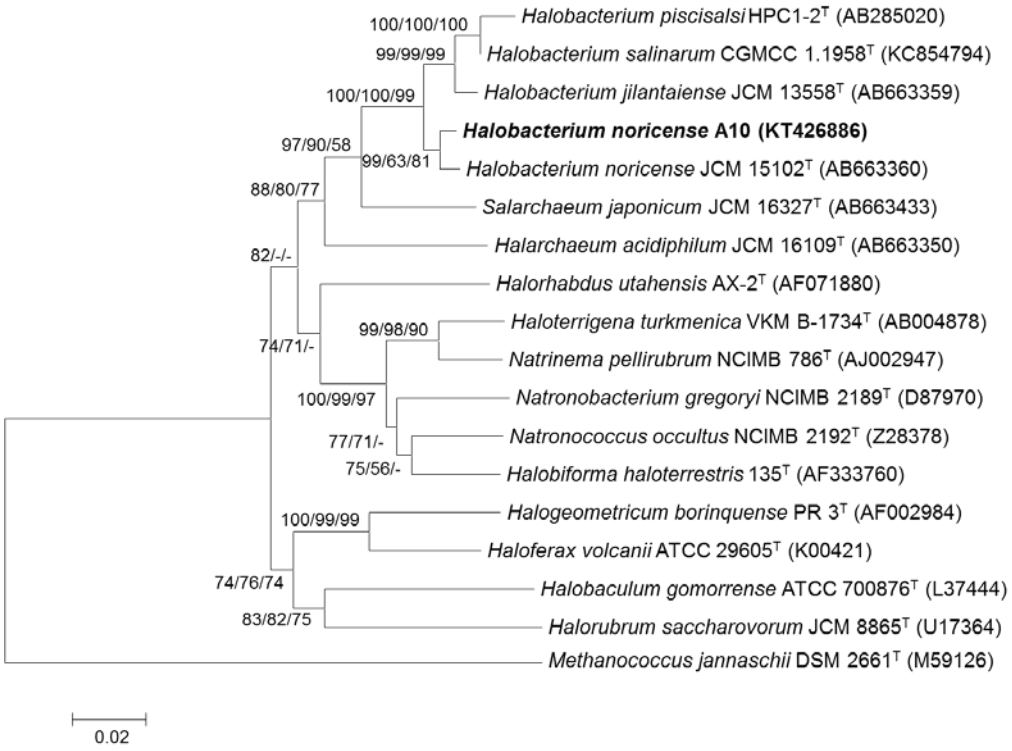
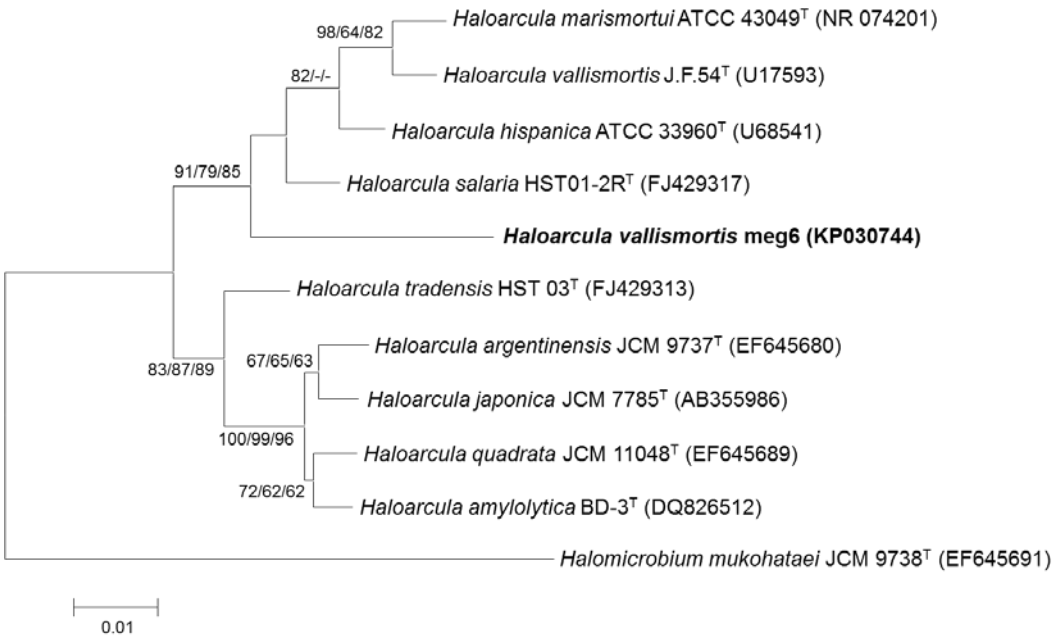


(D)



Chapter 2. *Halolamina sediminis* sp. nov., an extremely halophilic archaeon isolated from solar salt

1. Introduction

The genus *Halolamina* was firstly proposed by Cui *et al*. (2011) based on description of a novel species, *Halolamina pelagica* (type strain TBN21^T) with low 16S rRNA gene similarity (about 90 %) to other haloarchaea. Chemotaxonomic analyses showed that the genus *Halolamina* has distinctive characterizations including polar lipid composition. At the time of writing, the genus comprised four recognized species isolated from extremely saline environments: *H. peligica* (Cui *et al.*, 2011) from the marine solar saltern, *Halolamina rubra* (Cha *et al.*, 2014) from non-purified solar salt, and *Halolamina salifodinae* and *Halolamina salina* (Zhang *et al.*, 2013) from salt mine. Cells of species of the genus *Halolamina* are Gram-stain-negative, extreme halophilic (optimal NaCl concentration 20 %, w/v), strictly aerobic, and positive for catalase and oxidase activities (Cha *et al.*, 2014; Cui *et al.*, 2011; Zhang *et al.*, 2013). During estimation of the haloarchaeal diversity of a solar saltern in the western coastal area of the Korean Peninsula based on cultivation, one pleomorphic strain that phylogenetically belonged to the haloarchaea was isolated. In the present study, the taxonomic status of this novel archaeal strain, designated halo-7^T, was investigated by using polyphasic approach including determination of phenotypic and genotypic characterizations.

2. Materials and Methods

2.1 Sampling of solar saltern sediment and cultivation.

Strain halo-7^T was isolated from the sediment sample collected from a crystallizing pond of Gomso solar saltern in Jeollabuk-do Gunsan, Republic of Korea (salinity 17–23 %, temperature 35 °C and pH 7.8; 35° 35′ 44.6″ N 126° 37′ 02.5″ E, data from Park *et al.*, 2012). A 2 g solar saltern sediment sample was collected in a sterile conical tube and serially diluted in natural brine water (filter-sterilized, 0.22 μm pore size, Millipore) with 0.5% (w/v) yeast extract (Difco). The slurry mixture was aerobically incubated at 30 °C in the glass tubes and subcultured every 3 weeks with 1% (v/v) transferred to fresh DBCM2 medium. After three subcultures in DBCM2, the enrichment culture was spread onto solid DBCM2 agar plate (containing 1.5% (w/v) agar), and incubated at 37 °C for 2 weeks under aerobic condition. The isolates were successively transferred to the new DBCM2 agar plate, at least five times, and a single colony was finally designated halo-7^T.

2.2 Haloarchaea culture media

For the isolation of haloarchaea, two different medias were prepared: (i). DBCM2 medium [JCM medium no. 574; containing per liter: 833 ml MDS salt water, 1 ml $FeCl_2$ solution, 1 ml trace element solution, 0.25 g peptone (Oxoid), 0.05 g yeast extract (Difco), 5 ml 1 M NH_4Cl, 2 ml potassium phosphate buffer, 3 ml vitamin solution, 10 ml of 1 M sodium pyruvate solution], (ii). M372 [DSM medium no. 372; 20 g $MgSO_4 \cdot 7H_2O$, 5 g yeast extract, 5 g casamino acid, 3g trisodium citrate, 2 g KCl, 1 g sodium glutamate, 36 mg $FeCl \cdot 4H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$, 200 g NaCl per liter] were used.

2.3 Phylogenetic analysis

For phylogenetic analysis of strain halo-7^T, DNA was extracted using a commercial genomic DNA extraction kit (Geneall, Republic of Korea). The 16S rRNA gene was amplified by PCR from the chromosomal DNA with a universal archaeal primer set: forward primer 20F (5'-TTCCGGTTGATCCYGCCRG-3') and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (DeLong, 1992; Weisburg *et al.*, 1991). The PCR product was purified with a PCR purification kit (Cosmogenetech, Republic of Korea), and was sequentially sequenced by Cosmogenetech (Republic of Korea) using primers 20F, 340F, 958R, and 1492R (DeLong, 1992; Weisburg *et al.*, 1991). Full sequence of the gene was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database and aligned using the SILVA alinger (Pruesse *et al.*, 2012). 16S rRNA gene sequence similarities were calculated by using the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim *et al.*, 2012). Evolutionary distance were calculated by using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed based on the neighbor-joining (Saitou & Nei, 1987), minimum-evolution (Kidd & Sgaramella-Zonta, 1971) and maximum-likelihood (Felsenstein, 1981) methods by using MEGA6 software (Tamura *et al.*, 2013) with 1000 bootstrap replications for each.

2.4 Morphological and physiological characterization

The physiological characterization was performed with methods recommended in the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). For robust experimental reproduction, all tests were performed in triplicate unless stated otherwise. Gram staining was performed as described by Dussault (1955). Cell morphology and cell size, and the presence of flagella were determined by differential interference contrast microscopy (Axio Scope.A1; Carl Zeiss, Germany) and transmission electron microscopy (Tecnai G2 Sprite; FEI), respectively. Cell motility was examined by the modified-semisolid agar [supplemented with 25 % (w/v) sodium chloride] methods (Tittsler & Sandholzer, 1936). Cell lysis in distilled water and different salt concentrations [2-10 % NaCl (w/v), intervals of 2 %] was determined spectrophotometrically at 600 nm (DS-11+ spectrophotometer; DeNovix) and microscopically. Optimal growth conditions were determined using M372 or DBCM2 medium as a basal medium in the presence of 5–30 % (w/v) NaCl at 5 % intervals. The temperature range for growth was determined at 10, 15, 20, 25, 30, 37, 40, 45, and 50 °C in medium with optimal NaCl concentration (20–25 % w/v). The response to pH was determined by growth on M372 medium adjusted to pH 5.0–10.0 (at intervals of 0.5 pH units) by addition of the following buffers: 10 mM MES for pH 5.0–6.5, 10 mM Bis-Tris propane for pH 7.0–8.5, and 10 mM CAPS for pH 9.0–10.0. The requirement for magnesium ions for growth was determined using M372 medium made without $MgSO_4 \cdot 7H_2O$, and then supplemented with 10 mM Bistris propane and $MgSO_4 \cdot 7H_2O$ at concentrations of 0, 0.01, 0.02, 0.05, 0.5, 1, 2, 5, 100, or 500 mM.

For testing anaerobic growth with nitrate, L-arginine, trimethylamine *N*-oxide (TMAO) and DMSO (all at 5 g per mililiter) was tested using a 20 ml stoppered tube filled with M372 and MSG liquid medium and incubated in the dark at 37 °C for 4 weeks. Anaerobic growth was determined by turbidity (Oren *et al.*, 1997). The tests for catalase,

oxidase and urease activity, indole production and hydrolysis of casein and starch were examined as described by Benson (2002) using M372 medium as the basal medium. Hydrolysis of Tween 20, 40 and 80 was tested as described by Gonzalez *et al*. (1978) and hydrolysis of gelatin was assayed as described by Smibert & Krieg (1994). Production of H_2S was tested by growing the isolate and reference strains in a tube containing M372 liquid medium supplemented with 5 g per liter sodium thiosulfate and was determined using filter-paper strips impregnated with lead acetate (Cui *et al.*, 2007). The Methyl Red/Voges-Proskauer (MR/VP) reaction was determined according to Atlas (2004).

To test for growth of halo-7^T and reference strains on single carbon sources, M372 medium without casamino acids, yeast extract, sodium glutamate and sodium citrate was used. Various carbon sources to be tested were added to this modified medium at a concentration of 5 g per liter. Production of acid from carbohydrates (D-mannose, D-fructose, D-glucose, D-galactose and L-sorbose) was tested in unbuffered M372 medium supplemented with 5g per liter carbohydrate. Phenol red was used as an indicator to detect acid production, and compared with the initial pH and the pH of a control tube without added carbohydrates (Oren *et al.*, 1997). Antimicrobial susceptibilities were determined by the method of Gutiérrez *et al.* (2008) on M372 agar plates with antimicrobial compound discs for 4 weeks at 37 °C.

2.5 DNA G+C content, DNA-DNA hybridization and polar lipids analyses

Genomic DNA extracted for 16S rRNA gene amplification was used for determination of DNA G+C content. RNA in the DNA solution was removed by incubation with a mixture of RNase A and T1 (each, 20U per mililiter) at 30 °C for 1 h. The G+C content of the genomic DNA was determined according to Gonzalez & Saiz-Jimenez (2002).

DNA–DNA hybridization experiments were carried out with strain halo-7^T according to the method described by Ezaki *et al*. (1989). Genomic DNAs of strain halo-7^T, *H. salina* JCM 18549^T, *H. pelagica* JCM16809^T, *H. rubra* CBA1107^T and *H. salifodinae* JCM 18548^T were extracted by using a genomic DNA extraction kit (GeneAll) and used as probe DNA, respectively. Probe DNA was biotinylated with photobiotin and hybridized with single-stranded unlabelled genomic DNA fragments of reference or test micro-organisms.

Polar lipids of strain hlao-7^T and the reference strains were extracted and separated by one- and two-dimensional thin-layer chromatography on a silica gel glass plate (F254 silica gel-60 plates; Merck), then sprayed with the specific following reagents: sulfuric acid/ethanol (1:2, v/v) for total lipids, ninhydrin for amino-containing lipids, molybdenum blue for phospholipids and α-naphthol/sulphuric acid for glycolipids (Dittmer & Lester, 1964; Oren *et al.*, 1996; Xin *et al.*, 2000).

3 Results and Discussion

3.1 Phylogenetic analysis

Phylogenetic analysis indicated that strain halo-7^T clearly belongs to the genus *Halolamina* in the family *Haloferacaceae* within the order *Haloferacales* (Gupta *et al.*, 2015), as revealed by high bootstrap values (Fig. 2). Strain halo-7^T was related most closely to *H. salina* WSY15-H3^T (98.7 % 16S rRNA gene sequence similarity), *H. pelagica* TBN21^T (98.2 %), *H. rubra* CBA1107^T (97.4 %), and *H. salifodinae* WSY15-H1^T (97.4 %). In this study, based on 16S rRNA gene sequence similarity and phylogenetic analysis, the selected reference strains for comparison were obtained from the Japan collection of Microorganisms (JCM) for *H. salina* JCM 18549^T, *H. pelagica* JCM16809^T and *H. salifodinae* JCM 18548^T, and from the previous study for *H. rubra* CBA1107^T (Cha *et al.*, 2014).

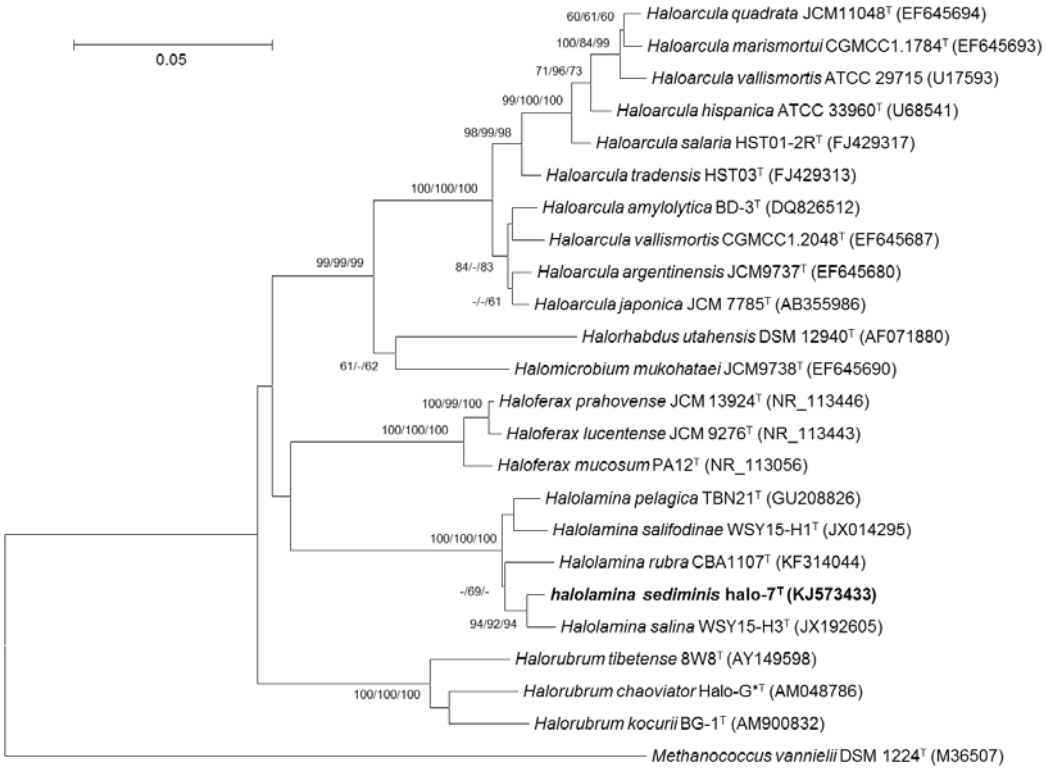


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between hlao-7^T, species of the genus *Halolamina* and representatives of related genera. *Methanococcus vannielii* DSM 1224^T served as an outgroup. Bootstrap values > 60% (based on 1000 replicates) from the neighbour-joining, maximum-likelihood and minimum-evolution methods, respectively, as indicated at branch points. GenBank accession numbers are shown in parentheses. Bar, 0.05 substitutions per nucleotide position.

3.2 Morphological and physiological characterization

Cells of strain halo-7^T were Gram-staining-negative, motile and pleomorphic with coccid (diameter 0.8–1.0μm) or rod-shaped (0.8–1.0 × 1.0–1.8 μm, width × length) morphology. Colonies were red-pigmented and had a smooth and rounded shape after incubation for 2 weeks on M372 agar. Strain halo-7^T grew in range of 15–30 % NaCl (optimal 20–25 %), at 25–45 °C (optimal 37–40 °C), pH 6.5–9.5 (optimal 7.0–8.0), and with 0.05–0.5 M Mg^{2+} (optimal 0.1–0.3 M). Magnesium ions were essential for growth. Strain halo-7^T lysed in distilled water and at concentrations below 10 % (w/v) NaCl. Nitrate, L-arginine, TMAO and DMSO were not utilized as electron acceptors for strain halo-7^T under anaerobic conditions. Strain halo-7^T was unable to hydrolyze casein, gelatin, Tween 20, 40 and 80. Cells were positive for catalase and oxidase activities, and starch hydrolysis, but negative for urease, *β*-galactosidase, ornithine decarboxylase, arginine and lysine activities, and H_2S and indole production. Strain halo-7^T produced acid from D-glucose and D-mannose. The strain was negative for MR/VP reaction. Strain halo-7^T used acetate, L-arginine, L-aspartate, citrate, fumarate, D-glucose, L-glutamate, glycerol, glycine, DL-lactate, L-lysine, L-malate, maltose, D-mannose, sorbitol and sucrose as sole carbon and energy source. Cells were sensitive to oxacillin (1 μg), sulfamethoxazole/trimethoprime (23.75/1.25 μg), and neomycin (50 μg) but resistant to streptomycin (10 μg), penicillin G (10 IU), clindamycin (2 μg), tetracycline (10 μg), gentamicin (10 μg), ampicillin (10 μg) and kanamycin (30 μg). Detailed results of phenotypic tests and nutritional traits of strain halo-7^T are presented in the species description, and are compared to related species of the genus *Halolamina* in Table 2. The optimum temperature, and pH and NaCl ranges for growth of strain halo-7^T are similar to those of other strains while the carbon source utilization of strain halo-7^T differs to those of the members of the genus *Halolamina*.

Table 2. Differential characteristics of strain halo-7^T and type strains of closely related species of the genus *Halolamina*.

Taxa: 1, halo-7^T (data from this study); 2, *H. salina* WSY15-H3^T (Zhang *et al.*, 2013); 3, *H. rubra* CBA1107^T (Cha *et al.*, 2014); 4, *H. salifodinae* WSY15-H1^T (Zhang *et al.*, 2013); 5, *H. pelagica* TBN21^T (Cui *et al.*, 2011). All taxa were Gram-stain-negative, catalase- and oxidase-positive, produced red-pigmented colonies, and required magnesium ions for growth. All strains lysed in distilled water and growth was not observed using TMAO, DMSO, L-arginine, and nitrate as alternative electron acceptors under anaerobic conditions. All strains were positive for utilization of L-arginine, L-glutamate, and D-glucose. All strains were negative for H_2S production, activity for urease, L-arginine, *β*-galactosidase, ornithine decarboxylase and lysine, MR/VP test, the formation of indole, hydrolysis of casein, gelatin and Tween 20, 40 and 80, acid production from D-fructose and L-sorbose, and utilization of D-fructose, lactose, mannitol, L-sorbose, succinate and D-xylose. +, Positive; –, negative.

Characteristic	**1**	**2**	**3**	**4**	**5**
Cell morphology	Pleomorphic	Pleomorphic	Rod-shaped	Pleomorphic	Pleomorphic
Motility	+	–	+	-	-
Temperature for growth (°C)					
Range	25–45	20–50	20–50	20–45	25–50
Optimum	37–40	37	37	37–42	37
pH for growth					
Range	6.5–9.5	6.0–7.5	6.0–9.0	6.0–9.0	5.5–9.5
Optimum	7.0–8.0	7	7	7.0–7.5	7.0–7.5
NaCl for growth (%, w/v)					
Range	15–30	10–30	15–30	10–30	10–30
Optimum	20–25	20	20–25	20–23	20–23
Mg^{2+} for growth (M)					
Range	0.05–0.5	0–2.5	0.0005–0.5	0–2.0	0–0.7
Optimum	0.1–0.3	0.02–2.5	0.1–0.2	0.1–0.5	0.01–0.05
Minimal NaCl conc. to prevent cell lysis (%, w/v)	10	8	12	9	8
Starch hydrolysis	+	–	–	–	+
Utilization of carbon source*					
L-Aspartate	+	–	–	–	–
D-Galactose	–	+	+	–	+
Glycine	+	–	+	–	–
DL-Lactate	+	–	–	+	+
L-Lysine	+	–	+	+	–
L-Malate	+	–	–	+	–

D-Mannose	+	–	+	–	+
Sucrose	+	+	–	+	–
Pyruvate	–	+	+	+	+
Acetate	+	–	–	+	+
Citrate	+	–	–	–	–
Fumarate	+	–	–	–	–
Glycerol	+	–	–	–	–
Maltose	+	–	–	–	–
Sorbitol	+	–	–	–	–
Acid production from D-glucose*	+	–	–	+	+
DNA G+C content (mol%)	68.0	66.2	65.1	65.4	64.8

* Data for reference strains obtained in this study.

3.3 DNA G+C content, DNA-DNA hybridization and polar lipids analyses

The G+C content of the genomic DNA of strain hlao-7^T was 68.0 mol%, a value that fell within the range for recognized species of the genus *Halolamina* (Table 2).

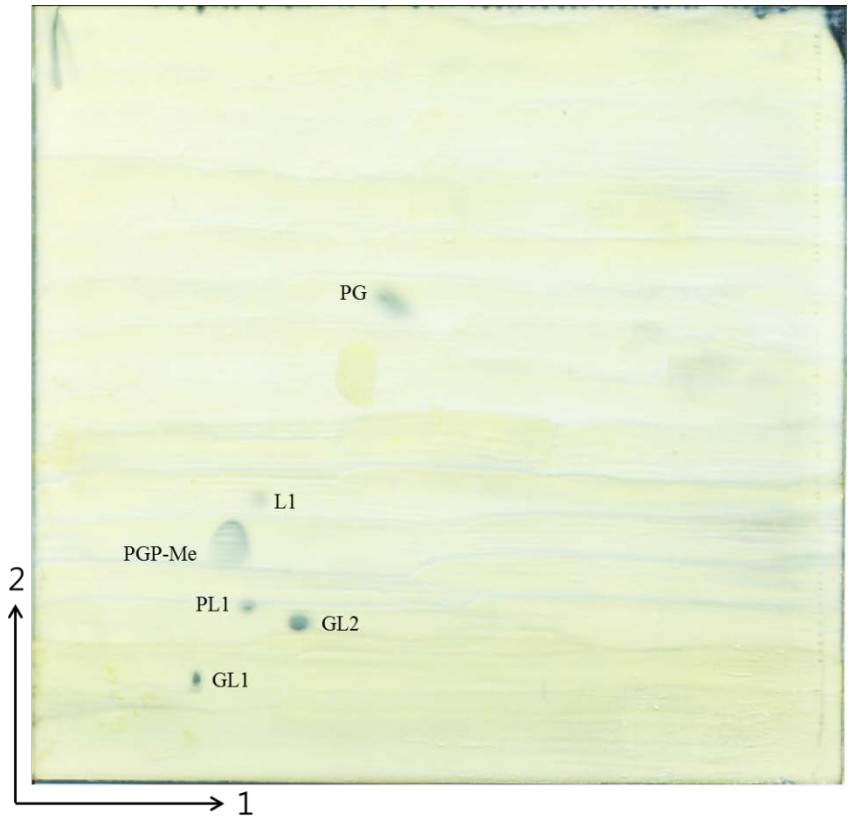
DNA-DNA relatedness values of strain halo-7^T with *H. salina* JCM 18549^T, *H. pelagica* JCM 16809^T, *H. rubra* CBA1107^T and *H. salifodinae* JCM18548^T were approximately 23.6, 12.6, 19.0 and 9.8 %, respectively (Table 3).

The polar lipids of strain halo-7^T comprised phosphatidylglycerol (PG), phosphatidylglyc erolphosphate methyl ester (PGP-ME), an unidentified phospholipid, an unidentified lipid and two unidentified glycolipids (Fig. 3). The polar lipid profile of strain halo-7^T was similar to that of other members of the genus *Halolamina*.

Table 3. DNA-DNA hybridization values for strain halo-7^T and the reference species

species	DNA-DNA hybridization (%) with:				
	halo-7^T	*H. salina* JCM 18549^T	*H. rubra* CBA1107^T	*H.salifodinae* JCM 18548^T	*H. pelagica* JCM 16809^T
H. sediminis halo-7^T	100	23.6	19.0	9.8	12.6

Fig. 3. Thin-layer chromatograms of the total polar lipids of strains halo-7^{T}. Two-dimensional thin-layer chromatograms of strains halo-7^{T}. The total polar lipids were detected using sulfuric 350 acid-ethanol. Abbreviations: PG, phosphatidylglyerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PL1, unidentified phospholipid; GL1-2, unidentified glycolipids; L1, unidentified lipid.



4. Description of *Halolamina sediminis* sp. nov.

Halolamina sediminis (se.di'mi.nis. L. gen. n. *sediminis*, of sediment).

Cells are motile, Gram-strain-negative, catalase- and oxidase-positive, and pleomorphic, short rod- or oval- shaped, 0.8–1.0 μm in width and 1.0–1.8 μm in length. Colonies are 0.2-0.4 mm in diameter, and are red-pigmented, smooth and round. Growth occurs in the presence of 15–30 % (w/v) NaCl (optimal 20–25 %), at 25–45 °C (optimal 37–40 °C), at pH 6.5–9.5 (optimal 7.0–8.0) and with 0.05–0.5 M Mg^{2+} (optimal 0.1–0.3 M). Cell lysis occurs in distilled water and the minimal NaCl concentration to prevent cell lysis is 10 % (w/v). Does not use nitrate, L-arginine, DMSO or TMAO as alternative electron acceptors under anaerobic conditions. Nitrate reduction to nitrite or the formation of gas from nitrate is not observed. H_2S is not produced from sodium thiosulfate. Indole is not produced from tryptophan. Does not hydrolyse casein, gelatin or Tween 20, 40 and 80. Negative result in tests for activities for urease, *β*-galactosidase, ornithine decarboxylase, L-arginine and lysine, and MR/VP reactions. The following substrates are utilized as single carbon and energy sources for growth: acetate, L-arginine, L-aspartate, citrate, fumarate, D-glucose, L-glutamate, glycerol, glycine, DL-lactate, L-lysine, L-malate, maltose, D-mannose, sorbitol and sucrose; L-alanine, D-fructose, D-galactose, lactose, mannitol, pyruvate, L-sorbose, starch, succinate and D-xylose are not utilized. Acid is produced from D-glucose and D-mannose. The polar lipids are PG, PGP-Me, an unidentified phospholipid, an unidentified lipid and two unidentified glycolipids.

The type strain is halo-7^T (=JCM 30187^T =CECT 8739^T) and was isolated from Gomso solar saltern, Republic of Korea. The DNA G+C content of the type strain is 68.0 mol%.

Summary

i. Majority of halophilic archaea are found in the diverse hypersaline environments like solar saltern, salt lake with very high salt concentration. The present study is about isolation and characterization of halophilic archaea from Gomso solar saltern known as a representative high salt environment in Korea. Finally, total seven strains were obtained identified on the basis of comparative similarity analysis for 16S rRNA gene sequence, physiological and biochemical traits. All halophilic archaea were belonged to *Haloruburm*, *Halogeometriucm*, *Halobacterium*, and *Haloarcula* genera.

ii. The phylogenetic analysis based on 16S rRNA gene sequences indicates that strain halo-7^T should be related to the genus *Halolamina*. However, strain halo-7^T differed from other species of the genus *Halolamina* with validly published names with regard to distinct phenotypic properties, such as pH, magnesium ions and salinity optimum and/or range for growth, the pattern of carbon sources utilized, and low DNA-DNA hybridization values (< 25 %). Thus, i propose that strain halo-7^T represents a novel species of the genus *Halolamina*, for which the name *Halolamina sediminis* sp. nov. is proposed.

iii. Basic information on microbial isolation techniques and related research in halophilic microorganisms from domestic halophilic environments is provided by this case study. It will be helpful in getting, useful indigenous halophilic archaea from a variety of extreme environmental conditions.

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고염 환경으로부터 극호염성 고균의 분리 및 특징 규명

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국문 요약

고균(archaea)은 지구상의 확립된 생물분류체계 3개의 도메인 중에 1개 이며, *Euryarchaeota, Crenarchaeota, Thaumarchaeota, Nanoarchaeota, Korarchaeota, Pacearchaeota, Woesearchaeota, Parvachaeota* 그리고 *Aigarchaeota* 으로 세분화되어 분류되고 있다. 주로 'Haloaraea'로 불리는 극호염섬 고균은 *Euryarchaeota* 문(phylum)에 속한다. 또한, 생장을 위하여 최소 1.5 M 이상의 염분을 필요로 한다. 대부분의 극호염성 고균은 고염환경으로 알려진 염호수(salt lake)와 천일염전(solar saltern)등을 비롯한 다양한 고염환경에서 서식하고 있다고 알려져 있다. 본 연구는, 한국의 대표적인 고염환경으로부터 분리배양을 통하여 극호염성 고균의 특성분석을 실시하였다. 염전 퇴적물로부터 극호염성 고균들을 분리하기 위하여, 고염배지를 제작하고 (20% NaCl), 다양한 극호염성 고균을 확보할 수 있었다. 분리한 극호염성 고균 중 신규성이 높은 고균 1 개와 미기록종 고균 7 개를 선별하였다. 분리 된 극호염성 고균들의 16S rRNA 유전자 서열을 통한 계통학 및 상동성 분석을 진행한 결과, *Halolamina* 속의 신규성이

높은 미생물 1종, *Haloarcula* 속의 미생물 1종, *Halobacterium* 속의 미생물 1종, *Halogeometricum* 속의 미생물 1종 그리고 *Halorubrum* 속의 미생물 4종을 각각 확보 할 수 있었다. 이들 극호염성 고균들은 모두 그람음성균이며, 붉은 색의 색소를 갖고 있으며, 증류수에서 용해 현상을 나타내었다. 그리고 분리된 모든 극호염성 고균들은 12-30 % (w/v, NaCl) 염분을 필요로 하며, nitrate를 전자 수용체로 사용한 혐기적 조건에서 성장이 관찰되지 않았다. 신규성이 높은 미생물은 Phenotypic, chemotaxonomic 그리고 phylogenetic 특징들을 통하여 기존에 밝혀진 종들과는 구별되는 것을 보여주었으며, *Halolamina sediminis*로 명명되었다. 이번 연구는 국내의 호염환경으로부터 극호염성 고균 관련 연구에 대한 기초적인 정보 및 분리기술을 제공하며, 국내의 다양한 극한환경에 서식하는 미생물 배양체의 확보를 통하여 국내 생물자원 확보에 기여할 것으로 기대한다.

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